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Formation of New Blood Vessels in the Heart Can be Studied in Cell Cultures*

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Summary

Controlled induction of the formation of new microvessels, i.e.,angiogenesis may be used one day to treat patient that for example had suffered a myocardial infarction. Experimental models of angiogenesis in the heart in vivo substantially stress the animal. We therefore developed a model of angiogenesis of the heart in vitro, where mouse and rat heart pieces are stimulated under controlled conditions in a three dimensional matrix. Capillary-like sprouts emerging in these cultures represent early to midterm steps of angiogenesis and can be quantified to study potential angiogenic compounds and underlying mechanisms.

Keywords: therapeutic angiogenesis, ischemia, neovascularisation, three-dimensional matrix, heart, capillaries

Background Information

Formation of new blood vessels, a new therapeutic concept

Angiogenesis is a process of creating new capillaries to increase the blood supply within an organ or tissue. It occurs for example in response to signals from an organ or tissue that is insufficiently oxygenated. In the heart, angiogenesis occurs in response to acute or chronic blockages (occlusions) of coronary arteries. These occlusions induce a state of hypoxia known as myocardial ischemia during angina pectoris or after a heart attack. In patients with coronary heart disease hypoxia and myocardial ischemia often affect the function of the heart and results in a significant morbidity and mortality. Stimulation of angiogenesis by pharmacological or molecular methods (therapeutic angiogenesis) would generate the formation of new coronary collaterals in the myocardium, i.e., small branches of arteries and veins that newly develop to bypass narrowed or blocked segments. Improving coronary collateral circulation would limit myocardial ischemia and is therefore a promising new concept (Cao et al., 2005).

Accordingly, substantial research resources are currently being invested into studying the therapeutic stimulation of angiogenesis. Multiple animal studies and some preliminary human studies have confirmed the concept of stimulation of collateral circulation by pharmacological or molecular methods. Current animal models of angiogenesis in vivo often use complete blockage by ligation or a gradual reduction of blood flow in a major coronary artery to induce hypoxia-driven angiogenesis. Many animals die due to myocardial infarction or cardiac rhythm disturbances during these – sometimes cumbersome – surgical procedures. Furthermore, myocardial ischemia is often quite a painful condition for the animals.

Studying angiogenesis in three-dimensional ell cultures

Models of angiogenesis *in vitro* are based on the capacity of activated endothelial cells (cells forming the walls of blood vessels) to invade three-dimensional substrates. These substrates (Matrix) may consist of collagen gels, plasma clot, purified fibrin, Matrigel, or a mixture of these proteins with others. It is possible to embed and culture pieces of blood vessels (vascular explants such as aortic rings) and to observe cells sprouting and forming capillary-like structures (CLS) (Nicosia and Ottinetti, 1990). These models allow the preservation of the vessel architecture during the *in vitro* assay, and thus are close to an "*ex vivo*" model (Kiefer et al., 2004). We have further developed the original angiogenesis *in vitro* model for aortic explants by Roberto Nicosia (Nicosia and Ottinetti, 1990), for the use with heart explants. Briefly, pieces (ca. 1 mm^3) of left ventricular myocardium of rat or mouse hearts are embedded in a fibrin-gel, overlayed with growth medium and angiogenic stimulants (agonists) and/or inhibitors (antagonist). These cultures are then incubated for 7 days. Active molecules are added again every third day, and oxygen saturation can be lowered down to 1% O_2. A single mouse heart allows assessing more than 20 different samples, each tested in octuplicates. After 7 days CLS quantification is carried out by visual scoring according to a scale that was defined by measuring the area of capillary sprouts by morphometric software (Fig. 1).

http://3r-training.tierversuch.ch/en/module_3r/sensitization_in-vitro/acd

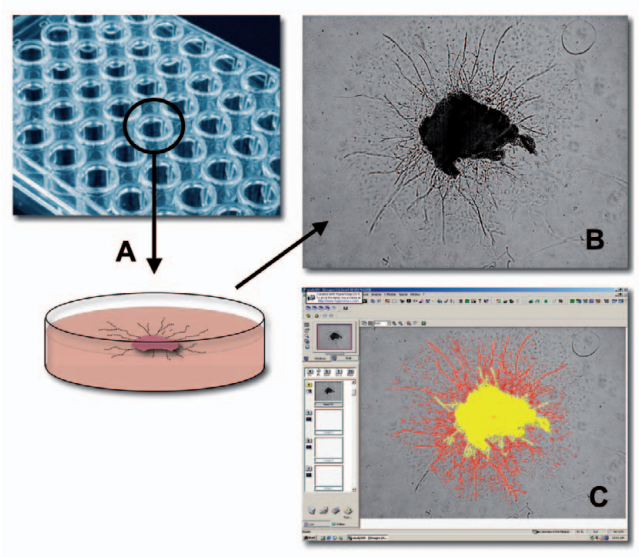

Fig. 1: Three-dimensional angiogenesis assay *in vitro*.
During *ex vivo* culture of heart or aortic tissue in fibrin gels, endothelial cells within the tissue are activated (by growth factors, hypoxia), limited proteolysis of surrounding matrix occurs, endothelial cells elongate, migrate and proliferate into the matrix and attract pericytes to stabilise the nascent capillary tube. Capillary tubes bifurcate and give rise to a primitive capillary tube-network (A). After 7 days of incubation in 48 well plates, pieces of heart are photographed digitally at 4X magnification (B). The total surface of sprouts and the piece of heart is measured (C). The fraction of heart and sprouts are set in relation to the total surface. Example: surface of sprouts is 81356 µm2 (red colouring) and surface of the heart is 152580 µm2 (yellow colouring); total surface is 233936 µm2; thus sprouts are 35% of the total surface corresponding to an angiogenic index of 4.

Reproducibility of the *in vitro* system

First we tested basic culture conditions to ensure reproducibility of the assay. In contrast to aortic explants forming CLS in serum-free conditions, CLS formation of heart explants requires fetal calf serum (5%) to ensure survival, whereas higher FCS concentrations mask the activity of angiogenically active molecules. In heart explants from mice younger than 8 weeks, CLS formed spontaneously – an unwanted response for testing the stimuli of angiogenic active factors. In 12-week-old adult mice however, virtually no CLS was formed under normoxia (standard culture condition, 20% O_2). Sex of the animals did not account for differences observed in CLS formation. Thus, for our future experiments, we chose adult mice, older than 12 weeks, to abolish the effect of active angiogenesis during adolescence of animals. Under hypoxia (1-3% O_2) however, CLS formation substantially increased, an established feature of angiogenesis, observed *in vivo* and *in vitro*.

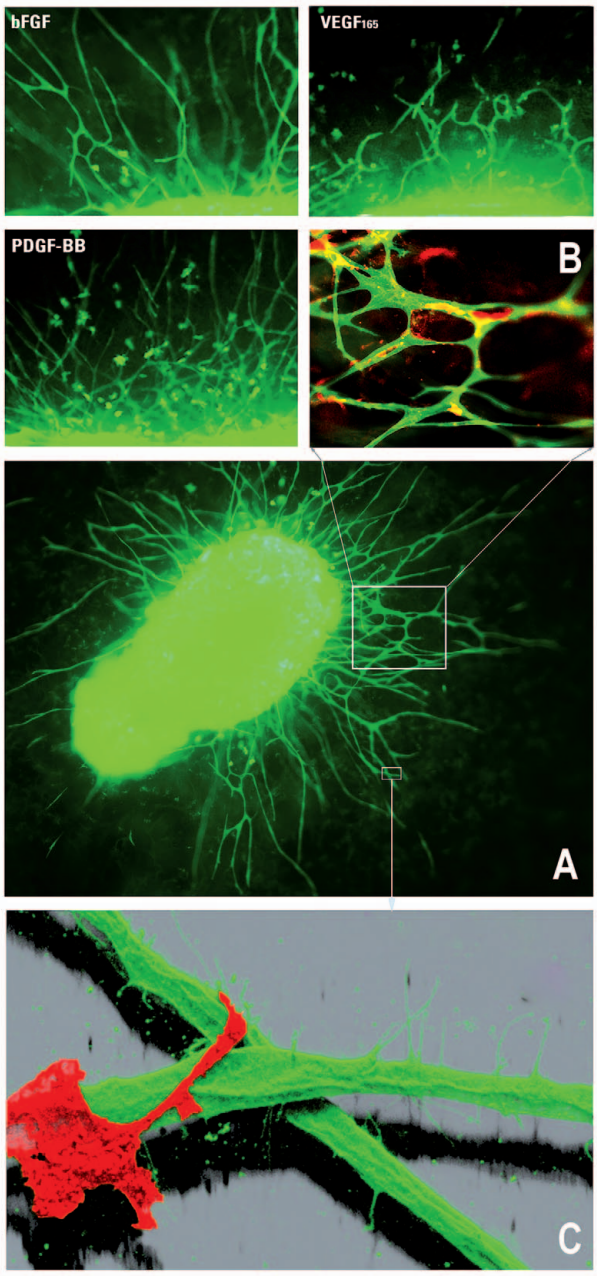

Fig. 2: Differential morphology of CLS can be observed after administration of different angiogenic molecules to mouse heart explants: basic Fibroblast Growth Factor – bFGF (10 ng/ml), Platelet-Derived Growth Factor B-dimer -PDGF-BB (10 ng/ml), Vascular Endothelial Growth Factor – VEGF (5 ng/ml).
A) In-gel characterization of CLS by FITC-coupled lectin G. simplicifolia (green fluorescent) for endothelial cells and B) magnification including Cy3-coupled anti alpha-smooth muscle actin-staining (red staining) for smooth muscle cell. C) Confocal micrograph of two crossing endothelial tubes. Tiny filpoodia reach out and sense the environment for neighboring endothelial tubes.

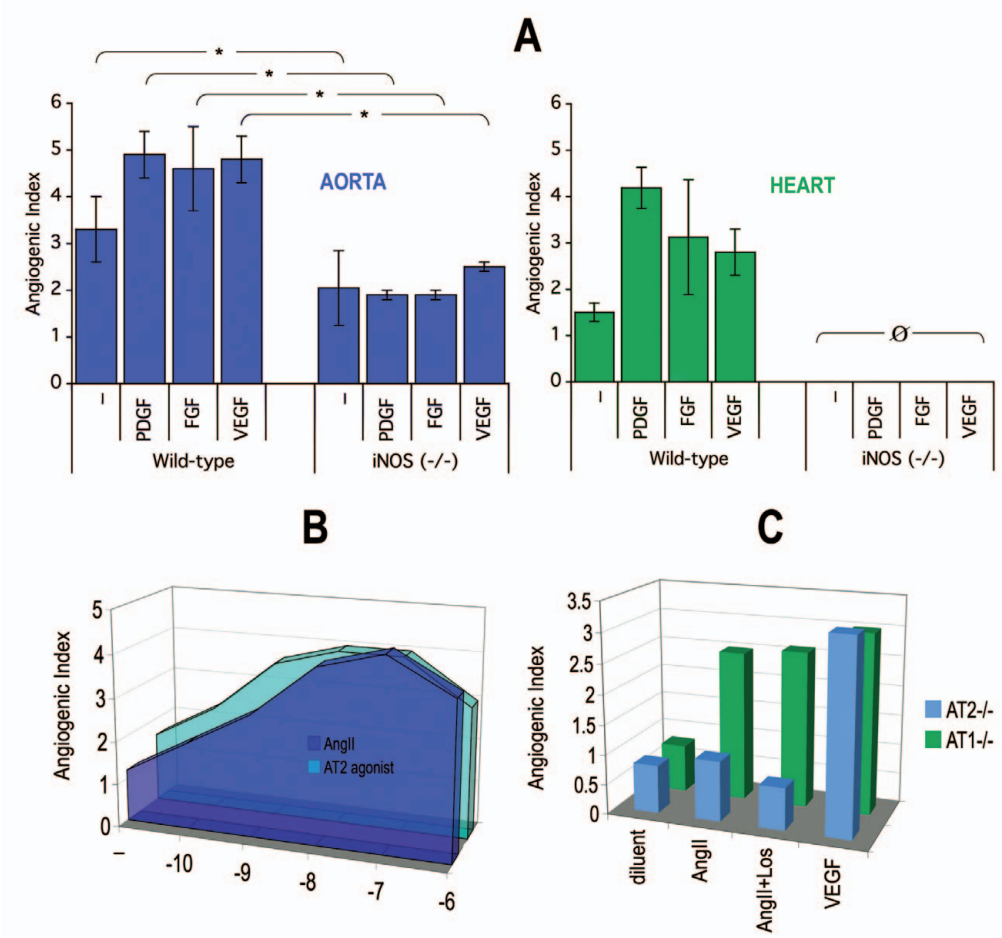

Fig. 3A: *In vitro* angiogenesis of the heart is abrogated under hypoxia in iNOS (–/–) mice.
In vitro angiogenesis of aortas (blue columns) and hearts (green columns) 7 days after addition of growth regulatory molecules: platelet-derived growth factor (PDGF)-BB (10 ng/ml), fibroblast growth factor (bFGF) (10 ng/ml) and vascular endothelial growth factor (VEGF)164 (5 ng/ml).
A standardized scale ranging from 0 to 6 indicates the degree of sprouting (angiogenic index). Data points given represent the mean of three experiments ± SEM. Ø – Sprouting undetectable; iNOS – inducible nitric oxide synthases.

Fig. 3B: Ang II induces dose-dependent sprouting *in vitro* in adult mouse hearts in hypoxia.
Pieces of mouse heart were stimulated with Ang II (blue graph) or AT2 agonist CGP-42112 (green graph) from 10-10 to 10-6 mol/L and incubated under hypoxia for 7 days. Data points represent the mean of 5 independent experiments. C. Ang II–induced angiogenesis is impaired in hearts from AT2-/- but not from AT1-/- mouse. Pieces of mouse heart from AT2-/- (blue columns) and AT1-/- mice (green columns) were stimulated with Ang II (10-7 mol/l) alone or in combination with AT1 blocker losartan (Los; 10-6 mol/l) and incubated under hypoxia for 7 days. VEGF164 (10 ng/ml) was used as a positive control. Data points represent the mean of 5 independent experiments.

Differential and *in vivo* like response toward angiogenic factors

Different classic angiogenic molecules induced diverse CLS patterns: Platelet Derived Growth Factor (PDGF-BB) induced outgrowth of a mixture of organized branched endothelial sprouts, unorganized single endothelial cells and pericytes/smooth muscle cells. In contrast, basic Fibroblast Growth Factor (ßFGF) induced mainly unbranched, elongated CLS. Vascular Endothelial Growth Factor (VEGF165) induced elongated CLS, and branching appeared complex (Fig. 2; ßFGF, PDGF-BB, VEGF165). Independent of the morphology induced by different agonists, outgrowing sprouts were composed of endothelial cells by more than 90% when outgrowing cells were subcultured and assessed by endothelial cell marker CD31, Dil-Ac-LDL. Double in-gel-staining with FITC-coupled lectin G. simplicifolia (green fluorescent) and Cy3-coupled antibody against a-smooth muscle actin (red fluorescence) revealed endothelial sprouts with single attached smooth muscle cell-like cells (pericytes) (Fig. 2A-2C). Pericyte attachment forming endothelial tubes have been observed *in vivo* and contributes to vessel remodeling, maturation and stabilisation. Also tiny filopodia, emerging from endothelial sprouts, can be observed by confocal microscopy (Fig. 2C). These filopodia sense neighbouring endothelial tubes and potentially guide network formation.

Nitric oxide as inducer of angiogenesis (Munk et al., 2006)

Nitric oxide (NO) promotes not only blood vessel relaxation and regulates vascular tone, but is critical for angiogenesis initiation and modulation (Sieber et al., 2001; Sumanovski et al., 1999). In this study we have investigated whether inducible NO synthase (iNOS) is required for angiogenesis *in vitro* of the heart versus angiogenesis originating from aorta..

We found that *in vitro* angiogenesis of the heart in mice lacking the iNOS gene (iNOS–/–) under hypoxia was totally abrogated and endothelial sprout formation could not be restored with classic angiogenic growth factors (Fig. 3A). In contrast, *in vitro* angiogenesis in aortas was still present, albeit reduced and without a response to angiogenic growth factors (Fig. 3A).

Thus, our results suggests that angiogenesis is regulated in an organ specific way and requires iNOS on the heart. iNOS-independent pathways may exist that can regulate angiogenesis in aorta and that are not present or less active in the hypoxic heart. This supports the need of organ specific models for angiogenesis and the concept that the angiogenic response in adult hypoxic hearts is far more restricted than in other organs.

Vasoactive peptides and their role in angiogenesis (Munk et al., 2007)

The vasoactive peptide Angiotensin II (Ang II) is a key regulator of blood pressure. Most of the Ang II cardiovascular effects, for example, vasoconstriction, are attributed to Angiotensin II'sAT1 receptor. AT1 is ubiquitously expressed, whereas the AT2 receptor is upregulated in response to ischemia and inflammation. Previous studies have shown that the AT2 receptor may interact with the bradykinin receptor, the B2 kinin receptor (BK2), during signaling. We have therefore investigated the mechanism of heart angiogenesis in response to Ang II under conditions of normoxia and severe hypoxia by dissecting the role of AT and BK receptor subtypes using the model of angiogenesis *in vitro*. We found that Ang II and AT2 receptor agonists dose-dependently induce angiogenesis *in vitro* of the heart and requires hypoxic conditions (Fig. 3B). However, Ang II was not angiogenic in mice hearts lacking AT2 receptor or, interestingly, the BK2 receptor, whereas no impairment of angiogenesis was observed in hearts from AT1-/- mice (Fig. 3C). We could corroborate these findings when using specific pharmacological antagonist against AT1, AT2, BK1 and BK2 receptors. We concluded that angiogenesis induced by Ang II requires signaling through the AT2 receptor and is mediated by an increase in BK production and activation of the BK2 receptor. Currently, we are using the assay to assess mechanisms of BK2-dependent angiogenesis.

A versatile *in vitro* model

Thus, a wide range of questions connected with therapeutic angiogenesis can be answered without the use of *in vivo* experiments: (1) Induction and repression of early to midterm morphogenic steps in angiogenesis can be investigated in mouse and rat hearts. (2) Pharmacological compounds can rapidly be screened using only a small number of animals. (3) Assays can be performed under controlled experimental conditions, such as under a distinct oxygen saturation. (4) The role of specific genes in heart angiogenesis can be investigated by using tissue from knockout mice.

The assay has gained attention by other research laboratories, particularly because of the possibility to use capillary sprouts for *in vitro* immunolocalization of proteins (Fig. 2).

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